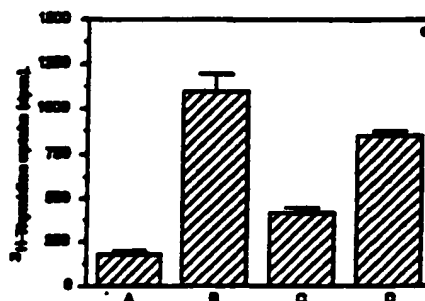
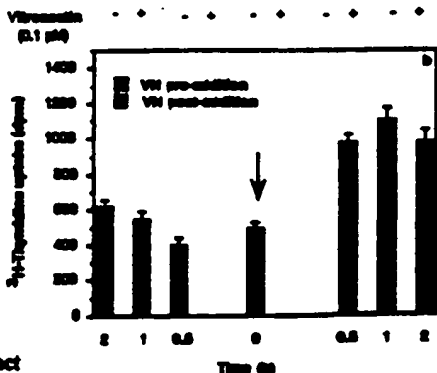
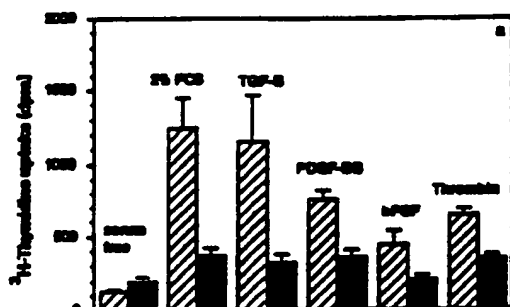




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(71) Applicants (for all designated States except US): THROMBOSIS RESEARCH INSTITUTE [GB/GB]; 9/11 Fulwood Place, Gray's Inn, London WC1V 6HQ (GB). MAX PLANCK GESELLSCHAFT [DE/DE]; Sprudelhof 11, D-6350 Bad Nauheim (DE).		(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC (European patent), MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, RU, SD, SE, SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent), US.	
(72) Inventors; and (75) Inventors/Applicants (for US only): WIJELATH, Errol [MY/GB]; DEMOLIOU-MASON, Catherine [GB/GB]; Thrombosis Research Institute, 9/11 Fulwood Place, Gray's Inn, London WC1V 6HQ (GB). HESS, Sibylle [DE/DE]; PREISSNER, Klaus, Theodore [DE/DE]; Max Planck Gesellschaft, Sprudelhof 11, D-6350 Bad Nauheim (DE). KAKKAR, Vijay, Vir [IN/GB]; Thrombosis Research Institute, 9/11 Fulwood Place, Gray's Inn, London WC1V 6HQ (GB).		Published With international search report.	

(54) Title: INHIBITION OF SMOOTH MUSCLE CELL PROLIFERATION BY VITRONECTIN



(57) Abstract

Vitronectin, an adhesion protein, acts as a stimulant for smooth muscle cell proliferation, a phenomenon which is known to cause atherosclerosis. The present invention relates to a method of inhibiting smooth muscle cell proliferation comprising administration of an agent which inhibits the action of vitronectin. Preferably, the vitronectin inhibitor is an antibody to vitronectin.

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smooth muscle cell proliferation, atherosclerosis, and other conditions involving smooth muscle cell proliferation. Compositions containing

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INHIBITION OF SMOOTH MUSCLE CELL PROLIFERATION BY VITRONECTIN

BACKGROUND OF THE INVENTION

This invention relates to the inhibition of smooth muscle cell proliferation and particularly to substances and compositions for use therein.

Background literature material, the disclosures of which are incorporated herein by reference, are referred to herein by parenthetical numerical citation in the text to the appended bibliography.

The hardening of human arteries caused by rapid and uncontrolled division of smooth muscle cells (hereinafter SMC) in the middle to outer arterial layers ("atherosclerosis") is a well recognised phenomenon and leads to narrowing of the arteries and increased chances of blood clot formation. This effect is a major cause of myocardial infarction in coronary patients and can for example lead also to gangrene in the case of lower limbs.

The association between atherosclerosis and growth factor-induced proliferation of vascular smooth muscle cells has prompted research towards the identification of negative modulators that may regulate normal SMC growth in vivo. Vascular SMC in culture are highly responsive to the chemotactic and mitogenic properties of platelet-derived growth factor (PDGF) and

it is believed that the paracrine and autocrine activity of this factor may play a major role in SMC migration from the media into the intima and in intimal SMC proliferation in atheroma development [3,4].

Recent studies [8] have shown that in atherosclerotic lesions there occurs deposition of a particular adhesion protein, namely vitronectin.

Vitronectin is one example of a so-called adhesion protein. It is a multifunctional glycoprotein present in blood and in various body tissues and plays a major role in cellular adhesion, immune defense mechanisms and haemostasis [1]. Vitronectin is involved particularly in focal adhesion and cell spreading, processes which are essential for wound healing and cellular movement [5,6,7].

Vitronectin shares the unique recognition sequence Arg-Gly-Asp (RGD) with the group of substrate adhesion proteins such as fibrinogen, fibronectin, van Willebrand factor and laminin. A common characteristic in binding of these proteins to cell surfaces is via the integrins [2], a family of heterodimeric membrane receptors which enable cells to anchor, migrate and make contact with other cells or extracellular matrix components, processes which are important for cell growth and tissue integrity.

As a result of in vitro studies to assess whether

vitronectin may affect SMC growth, we have now discovered that vitronectin acts as a growth inhibitory modulator of vascular smooth muscle cell proliferation.

This inhibitory effect of vitronectin extends also to the proliferation of 3T3-fibroblast cells.

The binding and effect of vitronectin on the response of SMC to PDGF and other mitogens have been characterised. In particular, the antagonistic effects of vitronectin on cell proliferation induced by a variety of known growth factors, notably that contained in 2% Fetal Calf Serum (FCS) and also PDGF and insulin-like growth factor (IGF-I), have been identified.

SUMMARY OF THE INVENTION

Accordingly, in one aspect the present invention provides a method of inhibiting smooth muscle cell proliferation, especially vascular smooth muscle cell proliferation, comprising administration of an effective amount of vitronectin.

In another aspect, the invention provides vitronectin for use as an antiproliferative agent.

The invention also provides pharmaceutical compositions comprising vitronectin together with a pharmaceutically acceptable diluent and/or carrier.

As described and discussed more fully hereinbelow, vitronectin may exist in either a folded

("native" - as present circulating in plasma) or an unfolded ("extended") conformation. For use in the present invention, the "extended" form is especially preferred, because of its superior antiproliferative activity.

When administering vitronectin as an antiproliferative agent in accordance with the invention, therefore, it will usually be the extended form which is supplied.

In accordance with this preferred aspect of the invention, the extended form of vitronectin is preferably multimeric, e.g. greater than the dimer and more preferably greater than the tetramer. This feature is preferred because of the superior antiproliferative activity of the higher molecular weight species.

However, because vitronectin in its native form is naturally present in the body, it is within the scope of the present invention to generate the extended form of vitronectin in situ by the action of a suitable agent which affects the equilibrium of the multimeric vitronectin species, for example proteoglycans, glycosaminoglycans and similarly acting agents and/or agents which are generated upon activation of the coagulation/fibrinolysis cascade.

Accordingly, in a further aspect of the present invention there is provided a method of inhibiting smooth muscle cell proliferation, comprising generating the extended form of vitronectin in situ by

administration of an agent which converts native vitronectin to its extended form.

Within the scope of this invention, the known vitronectin structure may vary by substitution, deletion or addition at one or more amino acid residues, so long as it retains essentially the same biological activity.

Vitronectin for use in accordance with the invention may be provided in any suitable form appropriate to the protocol of administration and/or the needs of a patient.

Particularly preferred is a composition comprising vitronectin together with one or more pharmaceutically acceptable carriers and/or diluents. Suitable carriers/diluents are well known in the art and include saline or other sterile aqueous media, optionally including additional components such as buffer salts and preservatives.

Alternatively, vitronectin for administration, together with or without pharmaceutically acceptable adjuncts as mentioned above, may be provided in lyophilised or freeze dried solid forms.

Administration may be via any suitable protocol. Preferably, administration is by intravenous injection or infusion, and may be systemic or topical.

In accordance with the present invention, administration of vitronectin as an antiproliferative

agent is in such an amount as to give the desired effective result of inhibiting SMC proliferation at the intended site. Thus, a quantity which constitutes an "effective" amount may depend upon various parameters, such as body weight of the patient, degree of inhibition required, intended site of activity, all of which will be well understood and appreciated by persons skilled in the art.

Generally, an amount of vitronectin will be administered which gives a concentration in plasma of from about 1 to about 100 mg ml⁻¹, more preferably from about 1 to about 10 mg ml⁻¹.

In a further aspect the present invention provides vitronectin for use in kits and assays, as well as such kits and assays, for example for screening individuals for those with a high risk of atherosclerosis. This may be achieved for example by exploiting the inhibitory effect of vitronectin on PDGF-induced SMC proliferation and observing the antagonistic effect of blood components, e.g. fibrinogen, on the degree of cell proliferation inhibition by vitronectin. (Such blood components bind vitronectin, thereby preventing it from performing its inhibitory function.)

A further example of the use of the present invention as a diagnostic tool is in the in vitro testing for whether a patient will react to

antiproliferative treatment with vitronectin.

The various aspects of the present invention will be further described by and better understood from the following detailed description, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a illustrates the inhibitory effect of vitronectin on vascular SMC proliferation induced by various growth factors;

Figure 1b illustrates the anti-mitogenic effect of vitronectin on PDGF-stimulated SMC when pre-added and post-added at various times;

Figure 1c illustrates the differences in SMC antiproliferative activity of the native and extended forms of vitronectin;

Figure 2a shows the dose-response profile of vitronectin on PDGF-induced DNA synthesis;

Figure 2b shows the electrophoresis profiles of native and extended vitronectin in the presence or absence of SDS;

Figure 2c is a plot of total binding of radioisotope-labelled vitronectin (native and extended forms) to SMC monolayers;

Figure 2d illustrates the inhibition of binding of radioisotope-labelled vitronectin (extended form) to

SMC in suspension by various peptides;

Figure 3a illustrates vitronectin inhibition of PDGF-induced DNA synthesis in human vascular SMC;

Figure 3b shows a Northern blot illustrating the absence of vitronectin inhibition of PDGF-induced mRNA expression of the c-fos proto-oncogene.

DETAILED DESCRIPTION OF THE INVENTION

In relation to Figures 1(a), 1(b) and 1(c), the following experimental procedures were used.

Experimental No. 1

Vascular SMC were isolated from sections of aortic or vein tissue removed during surgery and transferred into DMEM supplemented with 100 IU ml⁻¹ penicillin, 100µg ml⁻¹ streptomycin sulphate, 100 µg ml⁻¹ neomycin and 2.5 µg ml⁻¹ fungizone (Gibco). Dissected medial tissue was finally minced, rinsed and digested under constant agitation (18-20 h, 37°C) in DMEM 15 mM Hepes (Flow), 10% FCS with 0.1% collagenase Type II (Worthington) and 0.05% elastase Type I (Sigma). Cells were cultured in L-glutamine supplemented DMEM/ Hepes, 20% FCS plus antibiotics at 37°C in 10% CO₂ humidified incubators. FCS was reduced to 10% after the second passage. Cells grown to confluency were dissociated with 0.05% trypsin/0.02% EDTA and subcultured at a split ratio of 1:3. Experiments were

carried out between passage 2-8 with cells characterized as SMC by their morphology and SMC specific α -actin antigen expression. For DNA synthesis assays SMC were plated in 96-well plates at 1×10^4 cells/well in DMEM/Hepes supplemented with 10% FCS and antibiotics. 72 h later, cells were growth-arrested for 48 h by reducing FCS to 0.5%. Induction of DNA synthesis by growth activators was subsequently measured 24 h later (32 h for thrombin) in serum-free DMEM/Hepes by pulse labelling with 1 μ Ci [3 H]thymidine (Amersham) 6h prior to harvesting cells on a Brandel cell harvester using GF/B (Whatman) filters. Thrombin-specific activity was 3,000 U ml^{-1} . The extended form of human vitronectin was obtained after extensive dialysis of 6 M urea-treated "native" vitronectin [12]. Unless indicated otherwise cells were treated with vitronectin for 1 h prior to mitogen addition.

As shown in Figure 1a, treatment of growth-arrested, confluent human vascular SMC with 0.1 μ M human vitronectin inhibited DNA synthesis induced by various growth factors, namely: foetal calf serum (FCS) 2%; transforming growth factor -beta ($\text{TGF-}\beta$) 10 ng ml^{-1} ; PDGF-BB 10 ng ml^{-1} ; basic fibroblast growth factor (bFGF) 10 ng ml^{-1} (all from Bachem); or α -thrombin 5.0 U ml^{-1} (from Sigma). "-" represents in the absence of vitronectin; "+" represents in the presence thereof.

This inhibition occurred at a concentration of vitronectin (0.1 μM) well below the physiological range (3-6 μM ; 0.25-0.45 mg/ml of plasma) in circulation, and was greater than 50% (see the Figure for the exact percentage inhibition with respect to each growth factor) for all the mitogens tested. Similar results were obtained with aortic SMC. Vitronectin on its own had no effect on basal DNA synthesis.

Figure 1(b) illustrates SMC stimulation with 10 ng ml⁻¹ PDGF-BB in serum-free medium in the presence of 0.1 μM vitronectin (VN) added at the indicated times before (pre-addition) or after (post-addition) PDGF. The arrow indicates addition of PDGF and vitronectin simultaneously.

The anti-mitogenic response was observed only if vitronectin was added to the cells before or together with the growth factor, but not after the cells had been activated by the mitogen, suggesting a close association between mitogen-induced intracellular signal transduction and regulation of the cellular mechanisms involved in the anti-mitogenic response to vitronectin.

In sparsely plated, quiescent SMC, it was also found that vitronectin had no effect on mitogen-induction of DNA synthesis. This indicates that the response to vitronectin may depend on the differential expression of vitronectin receptor(s) and/or of other

cellular component(s) with cell growth.

Figure 1(c) illustrates the results of SMC incubation in serum-free medium (A) or stimulation with 10 ng ml^{-1} PDGF-BB (B) in the presence of $0.1 \text{ }\mu\text{M}$ "extended" vitronectin (C) or $0.1 \text{ }\mu\text{M}$ "native" vitronectin (D). Values represent the mean \pm SEM (n=4) of one of four representative experiments.

Although the concentration of native (i.e. folded) vitronectin in serum is high, serum-vitronectin is not anti-mitogenic suggesting that it is in an inactive state or associated with other serum components that may neutralize its anti-mitogenic activity. Only the extended (i.e. unfolded) form of vitronectin which is considered to be similar to that generated upon activation of the coagulation/fibrinolysis cascade or to that associated with extracellular matrix (ECM) components [9,10], appeared to have the preferential conformational state required for the transmission of this inhibitory effect. Purified human "native" vitronectin which is believed to be in the folded conformation [11] and constitutes the major form present in plasma [1], was considerably less active.

The specificity of the growth-inhibitory effect of vitronectin was confirmed by the attenuation of its activity in the presence of an anti-vitronectin antibody [12]. The results are shown in Table 1 below. Further,

the transmission of this effect via RGD-dependant interactions with receptor(s) on the cell surface was indicated in the inhibition by the cyclic RGD (cRGD)-peptide which specifically recognizes the vitronectin integrin receptor [13].

Table 1. Inhibition of the antiproliferative effect of vitronectin*

<u>Condition</u>	<u>DNA synthesis</u>
	<u>[³H]thymidine incorporation (dpm)</u>
No addition	180 ± 15
PDGF-BB (10 ng ml ⁻¹)	1200 ± 100
PDGF-BB + VN (0.1 µM)	420 ± 30
PDGF-BB + VN + cRGD (2 µM)	780 ± 38
PDGF-BB + VN + VN antibody (1/10,000)	982 ± 45

*SMC were stimulated with PDGF-BB in the presence of vitronectin (VN) and either the cyclic GPENGGRGDSPCA peptide [13] (cRGD) or a vitronectin specific polyclonal antibody [12]. DNA synthesis was measured as in Fig. 1. Neither the cRGD peptide nor the vitronectin antibody had an effect on basal or PDGF-induced [³H]thymidine incorporation. Results are the mean ± SEM (n=5) of a single experiment representative of two others.

The minimum concentration of cRGD that could be

used without causing the cells to detach within the time course required to measure DNA synthesis was 1.0 μ M. It could not be evaluated, therefore, whether the cRGD peptide is exerting its antagonistic effect by acting as a competitive inhibitor as in the case of cell adhesion to vitronectin [13]. Alternatively, the growth inhibitory effect of vitronectin may be transmitted via secondary interactions through other domains in addition to the RGD site, the kinetics of which may differ.

Such interactions that lead to a highly active vitronectin-integrin receptor association have been suggested recently from competition studies of [125 I]vitronectin binding to purified integrin receptor ($\alpha_v\beta_3$) and may explain the partial inhibition by the cRGD peptide observed here [14].

Since none of the other adhesive proteins tested, including fibronectin, collagen or fibrinogen were able to inhibit PDGF-induced DNA synthesis in confluent, quiescent SMC, it would suggest that the anti-mitogenic activity of vitronectin is transmitted via receptor(s) that recognize, in addition to the RGD site, specific regions of the vitronectin molecule such as the heparin binding domain.

In relation to Figures 2(a) to 2(d), the following experimental procedures were used.

Experimental No. 2

For the time course studies, human vein SMC plated in 48-well plates (3×10^4 cells per well) were growth arrested when confluent as in Fig. 1. After incubation with either form of [125 I]vitronectin (1.0×10^6 cpm ml^{-1}) in 175 μl serum-free DMEM medium/0.25% BSA for the time period indicated, cells were washed 3 times with DMEM/0.25% BSA, solubilized with 1N NaOH and counted. For [125 I]vitronectin binding to cells in suspension, confluent, growth-arrested cells were trypsinized as in Fig. 1 and incubated (2.5×10^4 cells per vial) with [125 I]vitronectin plus or minus competitor for 30 min. Binding was measured by filtration of cells on 3.0% BSA-pres soaked filters as in Fig. 1. Values represent the mean \pm SEM ($n = 3$) of one of three representative experiments. Both forms of vitronectin (native and extended) were radiolabelled and their specific activity ($1.5\text{--}2.5 \mu\text{Ci } \mu\text{g}^{-1}$) determined according to methods described previously [28].

Figure 2(a) illustrates the dose-dependent inhibition of PDGF-BB-induced DNA synthesis in SMC by vitronectin. SMC were stimulated with $10 \text{ ng } \text{ml}^{-1}$ PDGF-BB in the presence of the indicated concentrations of vitronectin. PDGF-induced [^3H]thymidine uptake in the absence of vitronectin was 1000 ± 110 dpm. Vitronectin alone had no effect on basal DNA synthesis.

The dose-response profile of vitronectin (0.1 nM-0.1 μ M) was biphasic, as shown in Figure 2(a). This biphasic response may reflect concentration-dependent differences in the anti-mitogenic or adhesive properties of the conformational states of oligomeric species formed as a result of exposure of epitopes in the extended form of vitronectin in agreement with the conformational lability/flexibility of this molecule [15,16].

The presence of such non-covalently linked multimers in the extended form was confirmed by the different electrophoresis profiles of native and extended vitronectin obtained under SDS conditions as compared to those in the absence of SDS. This is illustrated in Figure 2(b).

In this experiment native (lanes 1 and 3) and extended (lanes 2 and 4) [125 I]vitronectin were electrophoresed in 8% polyacrylamide gels in the presence (left panel) or absence (right panel) of 0.1% SDS (sodium dodecyl sulphate). Bars indicate the position of protein standards ($M_r \times 10^{-3}$). Arrows indicate the top of the separation gel. Samples (1×10^5 cpm) in 50 μ l Tris-buffer saline, 0.2% BSA with or without 1.0% SDS were loaded on each lane.

Figure 2(c) illustrates the time-dependent differential association of extended (e) and native (o)

[125 I]vitronectin 3.5-6.0 nM with SMC monolayers at 37°C. While native [125 I]vitronectin did not demonstrate appreciable binding, extended (multimeric) [125 I]vitronectin binding to cell surface receptors was considerably higher and appeared to reach steady state within 2 h. Since approximately 50% of extended [125 I]vitronectin binding to SMC monolayer was due to binding to ECM, the effects of cRGD and heparin on vitronectin binding to cell surface were assessed with cells in suspension. This is illustrated in Figure 2(d), which shows the inhibition of [125 I]vitronectin (extended form) (6.0 nM) binding to SMC in suspension at 37°C in the absence (A) or presence (B) of 100-fold molar excess unlabelled vitronectin; 1 μ M cRGD (C) or 10 μ g ml $^{-1}$ unfractionated heparin (Sigma) (D).

As Figure 2(d) shows, one hundred-fold excess of unlabelled vitronectin suppressed [125 I]vitronectin binding to SMC surface by more than 60%, indicating the specificity of the vitronectin-SMC interactions. Furthermore, [125 I]vitronectin binding was suppressed by 34% using the cRGD peptide (1 μ M) or by 50% using unfractionated heparin (10 μ g ml $^{-1}$), suggesting the involvement of both the RGD and heparin binding domains in the interactions of vitronectin with SMC cell surface receptors. In view of recent evidence on the role of heparan sulphate proteoglycans in the binding and

cellular responses to bFGF or TGF- β [17,18], it will be of interest to know whether the vitronectin effect is mediated via a similar dual receptor system involving a high-affinity RGD-dependent receptor and a low-affinity glycosaminoglycan-type binding site.

Alternatively, the inhibition of [125 I]vitronectin binding by heparin may be due to heparin binding and changes in the vitronectin conformation and also the monomer-oligomer equilibrium which may effect the concentration of active vitronectin species. Our preliminary studies have shown that the two molecules may neutralize each other's anti-mitogenic activity.

The relationship between vitronectin and heparin may be of importance in the regulation of cell growth, especially since heparin has been shown to inhibit mitogen and, in particular, PDGF-induced SMC proliferation [19,20].

As illustrated in Figures 3(a) and 3(b), vitronectin inhibits dose-dependent PDGF-induction of DNA synthesis in human vascular smooth muscle cells without affecting c-fos mRNA expression. Figure 3(a) shows the results of measurement of induction of DNA synthesis in SMC with increasing concentrations of PDGF-BB in serum-free medium in the absence (●) or presence (○) of 0.1 μ M vitronectin as in Figure 1. The results

represent the mean \pm SEM (n=4) of one of four representative experiments. Figure 3(b) shows a Northern blot of equal amounts (20 μ g) of total cellular RNA hybridized with a 2 kb c-fos probe or 0.53 kb G6PD probe. Growth-arrested, confluent SMC preincubated (1 h) without (lanes 1 and 2) or with (lane 3) vitronectin (0.1 μ M) in serum-free medium, were exposed for 30 min to 50 ng ml⁻¹ PDGF-BB prior to harvesting the cells for total RNA extraction.

Vitronectin was able to inhibit DNA synthesis over a range of concentrations of PDGF-BB (0.1-100 ng ml⁻¹), and at all concentrations the extent of inhibition did not vary (Fig. 3a). This effect was not due to binding and depletion of PDGF since vitronectin did not inhibit the induction of mRNA expression of the c-fos proto-oncogene by PDGF (Fig. 3b). These data also indicate that vitronectin may inhibit growth by affecting mechanisms other than those involved in the expression of early biochemical events induced by growth factors.

It has been shown recently that binding of plasminogen activator inhibitor-1 to vitronectin on ECM stabilizes its activity and prevents tissue or urokinase-type plasminogen activator from generating plasmin and subsequently matrix degradation [28]. Vitronectin, therefore, may prevent growth factor-

induced medial-SMC proliferation either directly via integrin/proteoglycan receptor activation or indirectly by preventing access of growth factors and chemo-attractants to SMC as well as cell migration via inhibition of ECM degradation by proteases. Generation of the extended form of circulating vitronectin at sites of vascular injury upon activation of the coagulation/fibrinolysis cascade, may be of physiological significance to limit the early proliferative stages of atheroma development or in intimal hyperplasia, since both of these events involve vascular SMC migration and proliferation.

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CLAIMS

1. A method of inhibiting smooth muscle cell proliferation, comprising administration of an effective amount of vitronectin.
2. A method according to claim 1, wherein the smooth muscle cells are vascular smooth muscle cells.
3. A method according to claim 1 or claim 2, wherein the vitronectin is the extended form thereof.
4. A method according to claim 3, wherein the extended form of vitronectin is multimeric.
5. A method according to any one of claims 1 to 4, wherein the vitronectin is administered in an amount such as to give a concentration in plasma of from 1 to 100 mg ml⁻¹.
6. A method according to claim 5, wherein the administration is in an amount such as to give a concentration in plasma of from 1 to 10 mg ml⁻¹.
7. A method according to any preceding claim, wherein the administration is by intravenous injection or infusion.
8. A method of inhibiting smooth muscle cell proliferation, comprising generating vitronectin in its extended form in situ by administration of an agent which converts native vitronectin to its extended form.
9. A method according to claim 8, wherein said agent

is selected from proteoglycans and glycosaminoglycans.

10. Vitronectin for use as an antiproliferative agent.

11. Vitronectin according to claim 10 which is in its extended form.

12. A pharmaceutical composition comprising vitronectin together with a pharmaceutically acceptable diluent and/or carrier.

13. A pharmaceutical composition according to claim 12, wherein the vitronectin is the extended form thereof.

14. Use of vitronectin in the preparation of a medicament for inhibiting smooth muscle cell proliferation.

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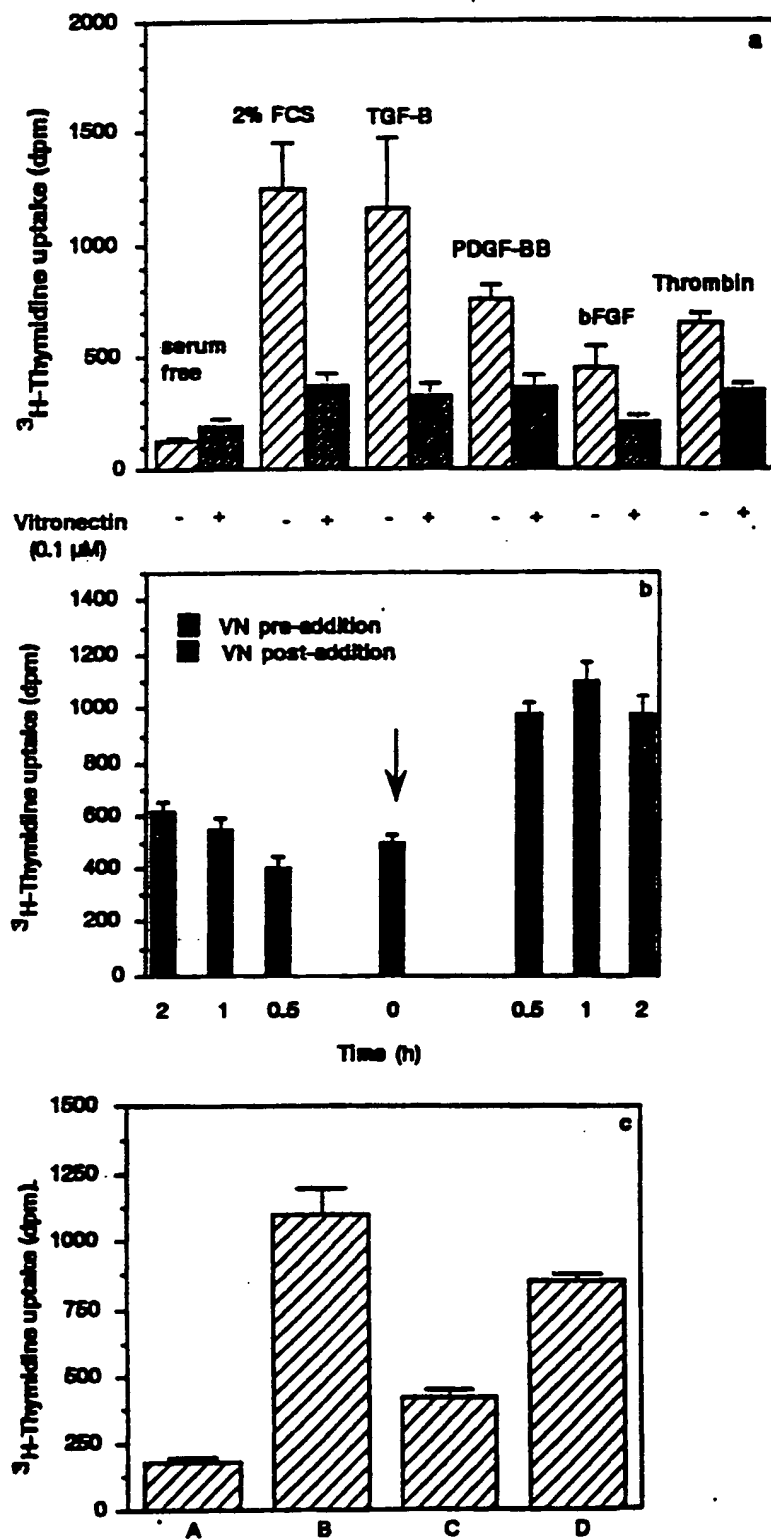


Figure 1

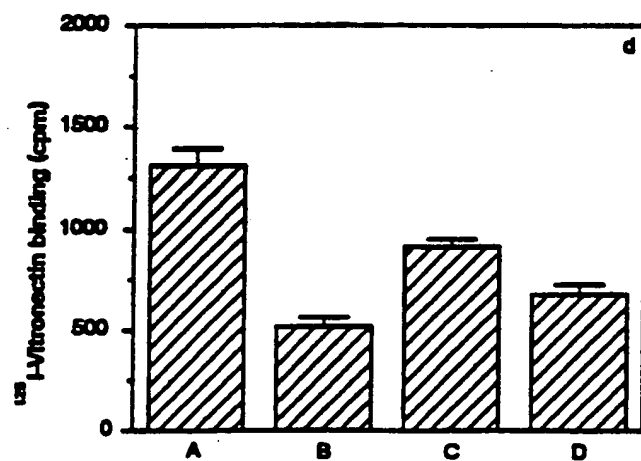
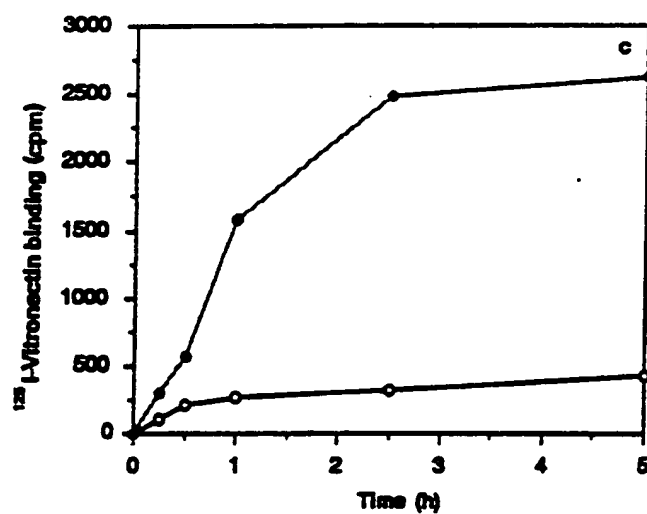
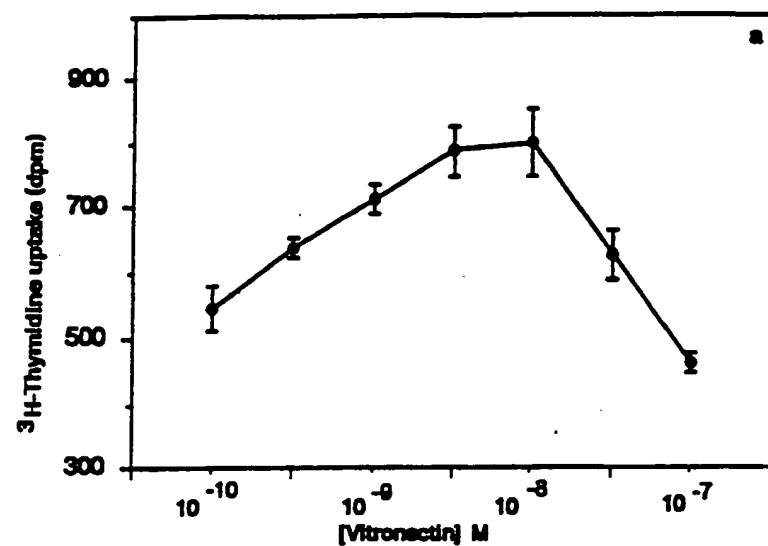


Figure 2

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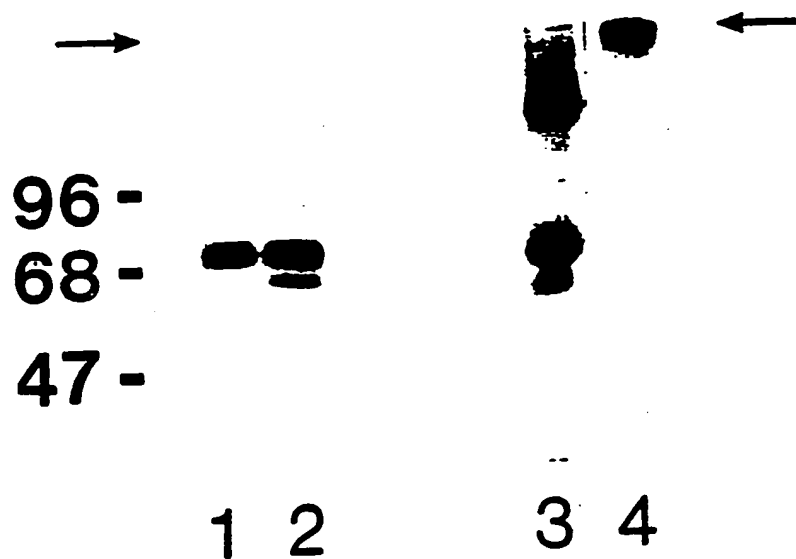


Figure 2b

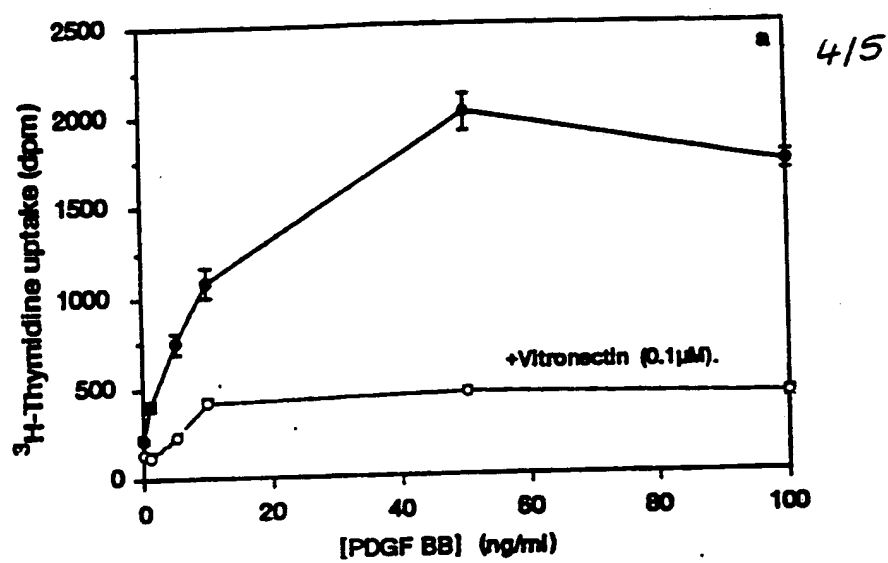


Figure 3a

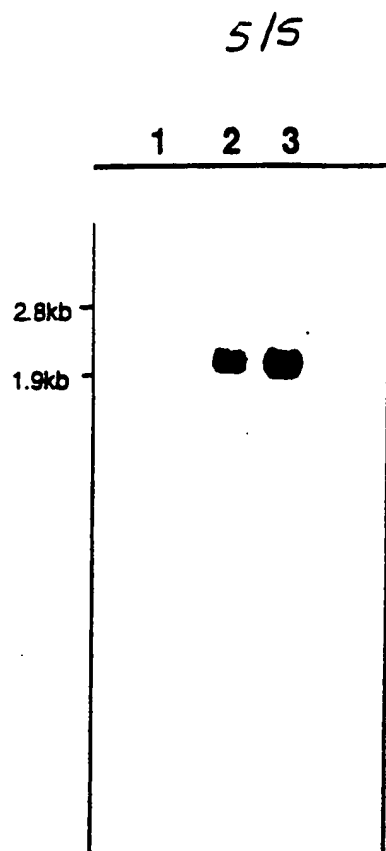


Figure 3b.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 92/00958

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC
 Int.C1.5 A 61 K 37/02 C 07 K 15/14

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System

Classification Symbols

Int.C1.5

A 61 K

C 07 K

Documentation Searched other than Minimum Documentation
 to the Extent that such Documents are Included in the Fields Searched⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	Journal of cellular Physiology, vol. 124, no. 1, July 1985, J.J. CASTELLOT et al.: "Effect of heparin on vascular smooth muscle cells. I. Cell metabolism", pages 21-28, see page 21, left-hand column, paragraph 2 - right-hand column, paragraph 2; page 23, right-hand column, paragraph 2 ---	1,2
A	EP,A,0341006 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 8 November 1989, see page 2, lines 37-47; page 5, lines 8-24,45-62 ---	1,2,5,6
A	EP,A,0292663 (RESEARCH DEVELOPMENT CORP. OF JAPAN) 30 November 1988, see page 3, lines 11-41; page 3, line 57 - page 4, line 7 --- -/-	12

¹⁰ Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

05-08-1992

Date of Mailing of this International Search Report

02.09.92

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

MONTERO LOPEZ B.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	EP, A, 0410006 (NISSHIN FLOUR MILLING CO., LTD) 30 January 1991, see page 3, line 23 - page 4, line 5; claim 1	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB92/00958

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-9
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-9 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9200958

SA 60006

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 18/08/92.
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0341006	08-11-89	US-A- 4945086	31-07-90
		JP-A- 2084402	26-03-90
EP-A- 0292663	30-11-88	JP-A- 1063600	09-03-89
		US-A- 5077393	31-12-91
EP-A- 0410006	30-01-91	JP-A- 2212433	23-08-90
		WO-A- 9009187	23-08-90

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